Immunochemical Quantification of Crossline as a Fluorescent Advanced Glycation Endproduct in Erythrocyte Membrane Proteins from Diabetic Patients With or Without Retinopathy

M. Yamaguchi[1], N. Nakamura*[1], K. Nakano[1], Y. Kitagawa[1], H. Shigeta[1], G. Hasegawa[1], K. Ienaga[2], K. Nakamura[2], Y. Nakazawa[2], I. Fukui[3], H. Obayashi[1,3], M. Kondo[1]

[1]The First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan
[2]Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Co., Ltd, Hyogo, Japan
[3]Department of Clinical Research, Kyoto Microbiological Institute, Kyoto, Japan

Crossline is a novel advanced glycation endproduct (AGE) which has both a crosslink and fluorescence similar to AGE-protein *in vivo*. To assess the association of AGEs to the development of diabetic retinopathy we developed a sensitive and specific enzyme-linked immunosorbent assay (ELISA) for crossline in blood samples and investigated the association of the development of retinopathy and erythrocyte membrane protein (EMP)-crossline concentrations in patients with Type 2 diabetes mellitus (Type 2 DM). Crossline formation in EMP exceeded that in haemoglobin and was detectable in normal EMP samples without pretreatment by this ELISA system. Mean (±SE) EMP crossline levels were elevated 1.6-fold in diabetic patients without retinopathy (7.6 ± 0.5 pmol mg^{-1}, $p < 0.005$), 2.2-fold in diabetic patients with non-proliferative retinopathy (10.5 ± 0.6 pmol mg^{-1}, $p < 0.001$) and 2.6-fold in diabetic patients with proliferative retinopathy (12.0 ± 0.6 pmol mg^{-1}, $p < 0.001$) compared with healthy control subjects (4.7 ± 0.5 pmol mg^{-1}). Type 2 DM patients with retinopathy had significantly higher EMP-crossline levels than those without retinopathy ($p < 0.005$). Our data suggest that elevated EMP-crossline concentrations are associated with the presence of retinopathy in patients with Type 2 DM and EMP-crossline measured by our ELISA may provide a useful marker for assessing the role of glycation in the development of diabetic retinopathy. © **1998 John Wiley & Sons, Ltd.**

***Diabet. Med.* 15: 458–462 (1998)**

KEY WORDS **crossline; advanced glycation endproducts; erythrocyte membrane; diabetic retinopathy; diabetic complication**

Received 11 September 1997; revised 1 December 1997; accepted 21 December 1997

Introduction

Maillard (non-enzymatic glycosylation) reactions of proteins by a reducing sugar, such as glucose, leads through early products such as Schiff's bases and Amadori adducts to advanced glycation endproducts (AGEs).[1,2] Excessive accumulation of AGEs has been implicated in diabetic complications, based on diverse biologic properties of AGEs, including protein crosslinking, cellular activation, growth promotion, and induction of vascular dysfunction.[3] AGE-adducts are yellow-brown pigments that can crosslink proteins and nucleic acids and their characteristic fluorescence (Ex 370/EM 440 nm) has been used widely as an indicator of the level of AGE-modified proteins. The structure of AGEs has been elucidated and some adducts, such as carboxymethyllysine,[4] pyrraline,[5] pentosidine,[6] and crossline[7] have been identified. Crossline was isolated as the main fluorescence AGE-product formed in a sugar-amine model system *in vitro* and has a fluorescence (EX 379/Em 463 nm) similar to that of protein fluorophores existing *in vivo*. In previous papers using specific anti-crossline antisera, we have shown that a crossline-like structure accumulates in the renal tissue and lens of rats with diabetes.[8,9] A simple and sensitive immunological assay for crossline in blood samples is necessary to investigate the association of diabetic complication and AGEs from a clinical viewpoint. Recently, we developed a highly sensitive competitive enzyme-linked immunosorbent assay (ELISA) system for crossline measurement in blood samples. In the

Abbreviations: AER albumin excretion rate, AGE advanced glycation endproduct, ELISA enzyme-linked immunoabsorbent assay, EMP erythrocyte membrane protein

* Correspondence to: Dr N. Nakamura, The First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamikyo-ku, 602 Kyoto, Japan

 CCC 0742–3071/98/060458–05$17.50

present study, we describe the establishment of an ELISA system for measurement of crossline in erythrocyte membrane proteins (EMP) and have investigated the association of diabetic retinopathy and EMP-crossline concentrations in patients with Type 2 (non-insulin-dependent) diabetes mellitus (Type 2 DM).

Patients and Methods

Subjects

Blood samples were obtained from 30 Type 2 diabetic patients without retinopathy; 24 Type 2 DM patients with proliferative retinopathy; 24 Type 2 DM patients with non-proliferative retinopathy, and 20 non-diabetic control subjects. All diabetic patients were diagnosed according to World Health Organization (WHO) criteria. Retinal examination was performed by an ophthalmologist through dilated pupils, with fluorescein angiology if clinically indicated. No retinopathy was diagnosed if no dot haemorrhages could be seen; proliferative retinopathy was diagnosed if neovascularization was present. To determine the status of diabetic nephropathy, we collected a single 24-h urine specimen to determine albumin excretion rate (AER) and classified subjects into three groups: normoalbuminuria (AER $\leq 20\ \mu g\ min^{-1}$), microalbuminuria (AER > 20 and $\leq 200\ \mu g\ min^{-1}$), and macroalbuminuria (AER $> 200\ \mu g\ min^{-1}$). Clinical characteristics of three groups of patients classified by retinopathy and healthy control subjects are shown in Table 1. The three groups were comparable for age, sex distribution, duration of diabetes, HbA_{1c}, and serum creatinine. There were no statistically (χ-square test) significant differences in incidence of micro- or macroalbuminuria among the three groups. None of the patients had uraemia.

Methods

Reagents

Anti-crossline antisera were prepared and characterized as reported previously.[8,9] Bovine serum albumin (BSA) conjugated with crossline as a coating antigen for the ELISA was prepared as reported previously.[9] *N*-acetyl-lysine derived crossline, used as a standard crossline, was prepared as reported previously.[7] Horseradish peroxidase (HPO)-conjugated anti-rabbit IgG was purchased from Bio-Rad (Richmond, California, USA).

Preparation of EMP

Freshly drawn blood, anticoagulated with heparin, was centrifuged at 2000 *g* for 10 min, and the buffy coat was aspirated along with the plasma. The red cells were washed three times with phosphate buffered saline (PBS), pH 7.6. The washed red cells were haemolysed in 10 to 20 volumes of cold 5 mmol l^{-1} Tris-HCl buffer containing 1 mmol l^{-1} EDTA and 0.2 mmol l^{-1} phenylmethylsulfonylfluoride, pH 7.8, and kept at 2°C for 60 min. The suspension was centrifuged at 20 000 *g* for 30 min. After carefully aspirating the viscous pellet of granulocyte debris and centrifuging once more, the ghosts were washed 4 to 5 times in a similar manner until white and finally suspended in 1 ml of PBS, pH 7.6. The protein concentration was determined by BCA protein assay (Pierce, Rockford, Illinois, USA) with BSA as a standard.

Western Blotting

To compare crossline formation in erythrocyte components, EMP and haemolysate supernatant (i.e. haemoglobin) samples were separated by electrophoresis on 4–20 % gradient SDS-polyacrylamide slab gels (SDS-PAGE) according to the procedure of Laemmli[10] and electrophoretically transferred to a PVDF membrane (Nihon Millipore Ltd, Yonezawa, Japan) according to the procedure of Towbin *et al.*[11] After transfer, membranes were blocked by incubating for 1 h in 4 % milk protein (Dai-Nippon Pharmaceutical, Osaka, Japan) at room temperature. After washing for 5 min with PBS containing 0.02 % Tween 20 (buffer A), membranes were incubated for 2 h in anti-crossline antiserum (1:500) at room temperature, washed with buffer A, incubated in HPO-conjugated anti-rabbit IgG (1:1000) for 2 h at room temperature, and washed with buffer A. The reaction products were visualized using 4-chloro-1-naphthol (4-CN) substrate (0.05 % 4-CN and 0.01 % H_2O_2 in PBS, pH 7.4).

Table 1. Clinical characteristics of three groups of NIDDM patients classified by retinopathy and controls

	NDR group	NPDR group	PDR group	Controls
Number (men/women)	30 (14/16)	24 (12/12)	24 (13/11)	20 (10/10)
Age (yr)	61.3 ± 5.5	62.1 ± 5.1	62.5 ± 4.3	61.0 ± 4.6
Duration of diabetes (yr)	15.7 ± 7.0	16.2 ± 7.2	16.5 ± 5.4	
HbA_{1c}(%)	7.9 ± 1.4	8.1 ± 1.5	8.2 ± 1.4	
Range of serum creatinine (mmol l^{-1})	45.2–113.1	56.5–113.1	56.5–124.4	
Incidence of microalbuminuria (%)	43.3	54.2	54.2	
Incidence of macroalbuminuria (%)	16.7	29.1	45.8	
Range of AER ($\mu g\ min^{-1}$)	210–252	208–274	206–292	

Data are expressed as mean ± SD.
NDR, no diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

Competitive ELISA for Crossline in EMP

Ninety-six-well microtitre plates (EIA plate I, Sanko Junyaku Co., Ltd, Tokyo, Japan) were coated with 100 μl per well of 0.3 μg ml^{-1} in coating buffer (50 mM carbonate buffer, pH 9.6) overnight at 4°C. Wells were washed twice with 200 μl buffer A, then blocked with 200 μl blocking buffer (4 % milk protein) at room temperature for 1 h. After washing with buffer A, 50 μl of sample (approximately 5 mg ml^{-1} EMP) in dilution buffer (0.4 % milk protein) was added, followed by 50 μl of anti-crossline antiserum (final dilution, 1:3000). Plates were incubated for 2 h at room temperature with shaking. After washing with buffer A, 100 μl of HPO-conjugated anti-rabbit IgG (1:2000) was added to each well and the plate was incubated for 2 h at room temperature. Plates were washed five times with buffer A and incubated with 100 μl of 0.04 % o-phenylendiamine dihydrochloride and 0.006 % H_2O_2 in phosphate-citrate buffer (pH 5.0) as the enzyme substrate. The reaction was stopped by addition of 100 μl of 1 mol l^{-1} H_2SO_4 and the colour reaction was read at 492 nm with an ELISA plate reader. The crossline contents of the samples were determined by fitting the absorbance of the samples to the standard curves obtained from the same competitive ELISA of *N*-acetyl-ysine crossline.

Statistical Analysis

Data are expressed as mean ± SE. The significance of the differences between study groups were analysed by a one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test using a StatView Software; $p < 0.05$ was considered significant. Linear regression analysis was performed by the linear least squares method and the correlation coefficients were determined.

Results

Formation of Crossline Among EMP Components

Figure 1 shows SDS-PAGE profiles and Western blot analyses with anti-crossline antiserum of EMPs and haemoglobin. On the Western blot analyses, an intense immunostaining band was found at a high molecular weight of between 100 and 250 kDa among the EMP components, whereas no immunostaining band or weaker band was observed among the haemoglobin components.

Establishment of ELISA System for Measurement of Crossline in EMPs

We established a highly sensitive competitive ELISA system for crossline analysis in EMPs with 30 ng well^{-1} of coating agent and diluted (1:3000) antiserum. The standard curve showed good linearity between 0.1 and 100 pmol well^{-1} of crossline (Figure 2). The lower detection limit of protein concentration in the EMP sample, without pretreatment as protease digestion from healthy subjects, was about 20 μg well^{-1} (Figure 2). The coefficients of intra- and inter-assay variations were 7.3 % ($n = 10$) and 9.8 % ($n = 7$), respectively.

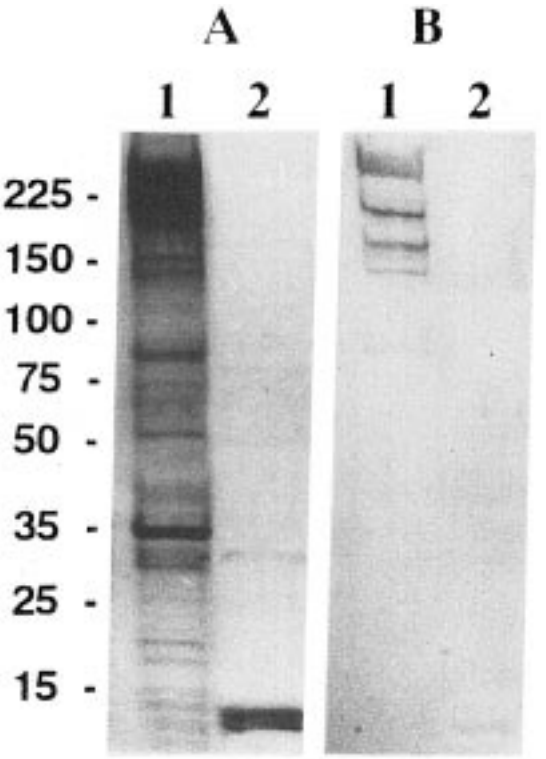

Figure 1. SDS-PAGE profiles (A) and Immunoblot analysis (B) with anti-crossline antiserum. Numbers on the left represent molecular weights (kD). Lane 1, ghost membranes fraction; lane 2, haemolysate supernatant fraction (haemoglobin)

Clinical Study

Figure 3 shows levels of crossline in EMP from study groups. The mean (±SE) EMP-crossline levels are elevated 1.6-fold in Type 2 DM patients without any retinopathy (7.6 ± 0.5 pmol mg^{-1}, $p < 0.005$), 2.2-fold in Type 2 DM patients with non-proliferative retinopathy (10.5 ± 0.6 pmol mg^{-1}, $p < 0.001$), and 2.6-fold in Type 2 DM patients with proliferative retinopathy (12.0 ± 0.6 pmol mg^{-1}, $p < 0.001$) compared to healthy control subjects (4.7 ± 0.5 pmol mg^{-1}). Further, Type 2 DM patients with retinopathy showed significantly higher mean EMP-crossline levels than those without retinopathy. No significant difference was found between the non-proliferative retinopathy group and the proliferative retinopathy group.

As shown in Figure 4, a moderate positive correlation between the levels of EMP-crossline and HbA_{1c} was observed in the present study ($r = 0.537$, $p < 0.01$).

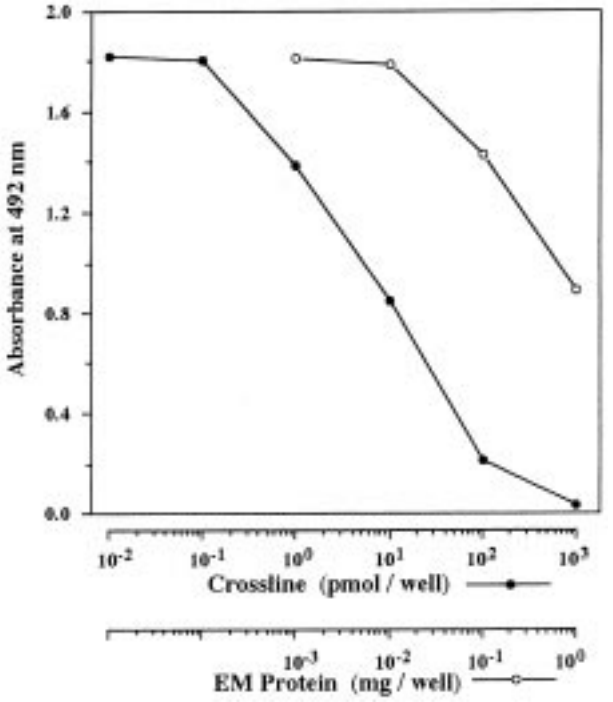

Figure 2. Typical calibration curve of the competitive ELISA for crossline (●) and dilution curve of crossline in unpretreated EMP from healthy subject (○)

Discussion

In our previous paper, we showed that crossline structures were formed at different rates in different proteins.[9] In the present study, first we investigated the difference of crossline formation in EMP and haemoglobin components by immunoblotting. The result demonstrated that the amount of crossline formation in EMP exceeded that in haemoglobin and that crossline in EMP can be measured by our ELISA system. Recently, Taneda and Monnier demonstrated that pronase E-digested plasma or AGE-modified protein increased 40 to 600-fold the immunoreactivity on ELISA for pentosidine.[12] In our similar study of crossline in serum and EMP measured by ELISA, the immunoreactivity of enzyme digested samples was increased 10 to 100-fold compared to non-digested samples (data not shown), however, the sensitivity of our ELISA system was sufficient to assay crossline in non-digested EMP samples (Figure 2). This ELISA system may provide a useful tool for clinical assessing of the role of AGEs in development of diabetic complications.

Increased glycation of EMP contributes to reduce deformability and membrane fluidity[13–15] and it has been suggested that these changes induce diabetic microangiopathy. Only a few attempts have been made at measuring glycation of EMP from diabetic patients. Miller *et al*.[16] and Scheicher *et al*.[13] reported that levels of early stage Maillard reaction products in EMP from diabetic patients exceeded those in EMP from normal control subjects. Our present study has demonstrated for the first time that crossline, one of major fluorescent AGEs, was present in EMP from diabetic patients at a higher concentration than in normal control subjects. More noteworthy is that Type 2 DM patients with retinopathy showed higher EMP-crossline levels than those without retinopathy, suggesting the possible involvement of EMP-crossline in the pathogenesis of diabetic retinopathy.

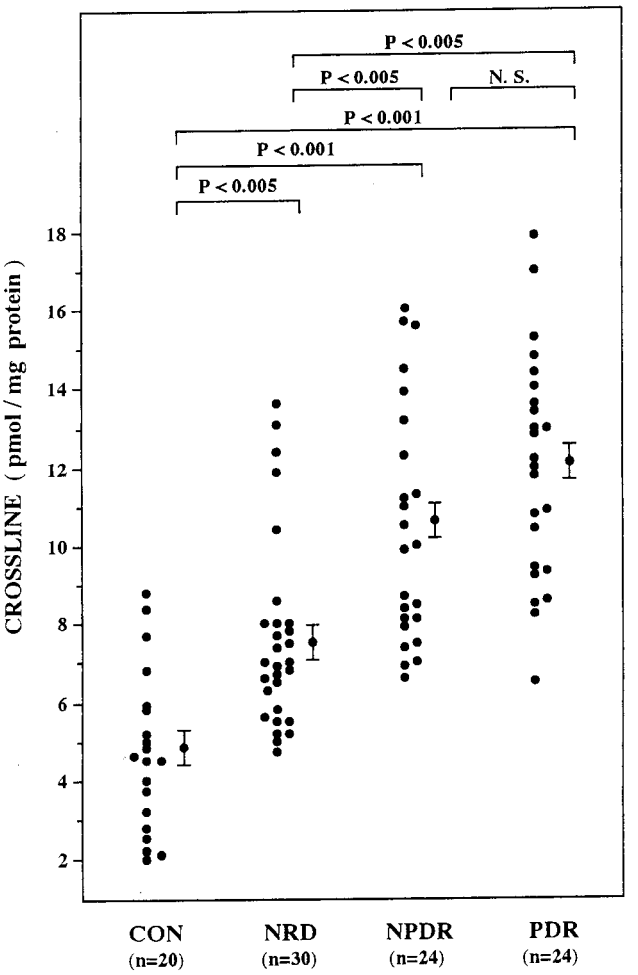

Figure 3. Distribution of crossline values among EMP from control (CON), no retinopathy diabetic (NRD), non-proliferative diabetic retinopathy (NPDR), and proliferative diabetic retinopathy (PDR) obtained by competitive ELISA. Bar shows mean ± SE

There was a moderate positive correlation between the levels of EMP-crossline and HbA_{1c}. However, HbA_{1c} levels did not correlate with diabetic retinopathy status. A similar finding was noted by both Monnier *et al*.[17] and McCance *et al*.[18] HbA_{1c} is formed slowly and nearly irreversibly and reaches a certain point which correlates to mean glucose level. The level of HbA_{1c} best reflects the average glucose concentration over the previous 2–3 month period.[19] In contrast, AGEs remain irreversibly attached and continue to accumulate over the life-span of the protein. Therefore, EMP–AGEs may serve as an ideal integrator of blood sugar, reflecting the full 60-day half-life of the erythrocyte.[20] Although the biological activity of crossline has not been demonstrated, increased AGEs in EMP could be an important factor in the

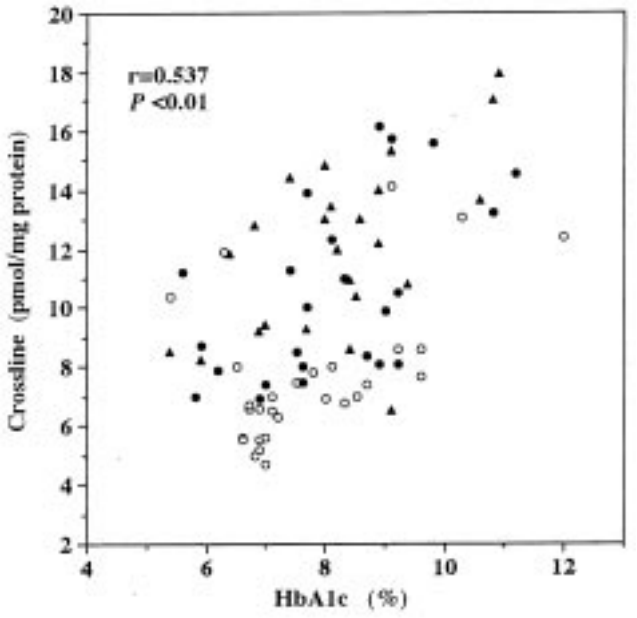

Figure 4. Correlation between amounts of EMP crossline and HbA_{Ic} for 30 NIDDM patients without retinopathy (○), 24 NIDDM patients with non-proliferative retinopathy (●), and NIDDM patients with proliferative retinopathy (△)

development of diabetic micro- and macrovasculopathy. It has been demonstrated that AGEs on erythrocyte cell surface induce receptor-mediated phagocytosis by macrophages[21] and that AGEs on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor that leads to oxidant stress in the vasculature.[22]

In conclusion, our data suggest that elevated EMP-crossline concentrations are associated with the development of retinopathy and EMP-crossline measured by our ELISA system may provide a useful marker for assessing role of glycation in the development of diabetic retinopathy. Prospective studies are needed to evaluate the elevated EMP-crossline concentrations as a marker of progression or prediction to diabetic retinopathy.

References

1. Monnier VM, Cerami A. Nonenzymatic browning *in vivo*: possible process for aging of long-lived proteins. *Science* 1981; **211**: 491–493.
2. Brownlee M, Vlassara H, Cerami A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 1984; **101**: 527–537.
3. Vlassara H, Bucala R, Striker L. Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* 1994; **70**: 138–151.
4. Ahmed MU, Thorpe SR, Baynes JW. Identification of *N*-ε-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 1986; **261**: 4889–4894.
5. Njoroge FG, Sayre LM, Monnier VM. Detection of D-glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr Res* 1987; **167**: 211–220.
6. Sell DR, Monnier VM. Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentosee in the aging process. *J Biol Chem* 1989; **264**: 21597–21602.
7. Nakamura K, Hasegawa T, Fukunaga Y, Ienaga K. Crossline A and B as candidates for the fluorophores in age- and diabetes-related crosslinked protein and their diabetes produced by Maillard reaction of α-N-acetyl-L-lysine with d-glucose. *J Chem Soc Chem Commun* 1992; **14**: 992–994.
8. Ienaga K, Kakita H, Hochi T, Nakamura K, Nakazawa Y, Fukunaga Y, *et al*. Crossline-structure accumulates as fluorescent advanced glycation endproducts in renal tissue of rats with diabetic nephropathy. *Proc Japan Acad* 1996; **72B**: 79–84.
9. Obayashi H, Nakano K, Shigeta H, Yamaguchi M, Yoshimori K, Fukui M, *et al*. Formation of crossline as fluorescent advanced glycation end product *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 1996; **226**: 37–41.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
11. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. *Proc Natl Acad Sci* 1979; **76**: 4350–4354.
12. Taneda S, Monnier VM. ELISA of pentosidine, an advanced glycation end product, in biological specimens. *Clin Chem* 1994; **40**: 1766–1773.
13. Schleicher E, Scheller L, Wieland OH. Quantitation of lysine-bound glucose of normal and diabetic erythrocyte membranes by HPLC analysis of furosin [ε-N(L-furoylmethyl)-L-lysine]. *Biochem Biophys Res Commun* 1981; **99**: 1011–1019.
14. Bryszewska M, Szosland K. Association between the glycation of erythrocyte membrane proteins and membrane fluidity. *Clin Biochem* 1988; **21**: 49–51.
15. Watala C, Zawodniak M, Bryszewska M, Nowak S. Nonenzymatic protein glycosylation. I. Lowered erythrocyte membrane fluidity in juvenile diabetes. *Ann Clin Res* 1985; **17**: 327–330.
16. Miller JA, Gravallese E, Bunn HF. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. *J Clin Invest* 1980; **65**: 896–901.
17. Monnier VM, Vishwanath V, Frank KE, Elmets CA, Dauchot PJ, Kohn RR. Relation between complications of type 1 diabetes mellitus and collagen-linked fluorescence. *N Engl J Med* 1986; **314**: 403–408.
18. McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, *et al*. Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 1993; **91**: 2470–2478.
19. Bunn HF, Haney DN, Kamin S, Gabbay KH, Gallop PM. The biosynthesis of human hemoglobin A_{1c}: slow glycosylation of hemoglobin *in vivo*. *J Clin Invest* 1976; **57**: 1652–1659.
20. Makita Z, Vlassara H, Rayfield E, Cartwright K, Friedman E, Rodby R, Cerami A, Bucala R. Hemoglobin-AGE: a circulating marker of advanced glycosylation. *Science* 1992; **258**: 651–653.
21. Vlassara H, Valinsky J, Brownlee M, Cerami C, Nishimoto S, Cerami A. Advanced glycosylation endproducts on erythrocyte cell surface induce receptor-mediated phagocytosis by macrophages. *J Exp Med* 1987; **166**: 539–549.
22. Wautier JL, Wautier MP, Schmidt AM, Anderson GM, Hori O, Zoukourian C, *et al*. Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc Natl Acad Sci* 1994; **91**: 7742–7746.